Protective Effect of a Vitamin E Analog, Phosphatidylchromanol, Against Oxidative Hemolysis of Human Erythrocytes

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ABSTRACT: The protective effect of a vitamin E analog, phosphatidylchromanol [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman; PCh], against oxidative hemolysis of human erythrocytes was examined and was compared with those of vitamin E (α-tocopherol) and 2,2,5,7,8-pentamethyl-6-chromanol (PMC). These three compounds at 50 μM protected the erythrocytes from hemolysis, when erythrocyte suspension (10%, vol/vol) was incubated with a water-soluble radical generator, 2,2′-azobis(2-amidinopropane)-dihydrochloride (75 mM). When erythrocyte suspension was oxidized after pretreatment with these compounds (50 μM) for 30 min followed by washing, PCh protected about 54% of erythrocytes from the hemolysis, while α-tocopherol protected only about 16% of the cells and PMC did not show any protective effect. During preincubation, α-tocopherol, PMC, and PCh were incorporated into the cells at the concentration of 12.6, 3.7, and 16.3 nmol/mg protein, respectively. Moreover, PCh was found in the ghost membrane fraction at a 20% higher level than α-tocopherol, and no PMC was detected in this fraction. These results indicate that phosphatidyl group in PCh acts as an excellent carrier of chromanol moiety into cells as well as an anchor within membranes more efficiently than phytyl group in α-tocopherol. PMC seems to be slightly anchored within membranes because of the lack of hydrophobic side chain. The excellent antihemolytic activity of PCh is likely to be caused by its accumulation within erythrocyte membranes.
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Increasing evidence suggests that free radical-induced oxidative damages lead to various pathological events including coronary heart disease, cancer, and aging (1). In particular, lipid peroxidation in biological membranes has attracted much attention in relation to the deterioration of membrane structure and impairment of enzymatic functions (2). Much interest exists in the possibility that antioxidants reduce the risk of such degenerative diseases by inhibiting free radical-induced oxidative damage (3). Therefore, several studies have examined both natural and synthetic antioxidants for the inhibition of lipid peroxidation in membrane systems.

In phospholipid bilayers of biomembranes, the primary defense against free radical-induced oxidative damages involves vitamin E (α-tocopherol) (4,5), and the presence of specific binding sites for α-tocopherol in erythrocyte membranes was demonstrated (6,7). α-Tocopherol consists of two structural domains. One is a chromanol group, responsible for radical scavenging ability, and the other is phytyl side chain, which is required for anchoring the chromanol group within phospholipid bilayers (8). It has been accepted that the phytyl side chain of α-tocopherol facilitates the incorporation of α-tocopherol into biomembranes and arranges its chromanol group on a suitable site for scavenging chain-carrying lipid peroxyl radicals in phospholipid bilayers (9,10). Thus, the phytyl side chain of α-tocopherol has an important role in its antioxidant activity in the membranes.

In previous papers, we described a new synthetic vitamin E analog, phosphatidylchromanol [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′8′-tetramethyl-6′-hydroxychroman (PCh), Scheme 1], which contains phosphatidyl moiety as the substitute for phytyl chain (11). Its antioxidant abilities in bulk oils and phospholipid bilayers were also examined by comparing them with those of α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) (11–14). PCh is expected to act as an effectively protective reagent against oxidative cellular injury, because phosphatidyl group seems to possess high affinity for cellular phospholipid bilayers and has been actually regarded as a suitable and nontoxic carrier of pharmaceuticals into cells (15). The aim of this study is to elucidate the action of PCh as a protective reagent against oxidative damages mediated by lipid peroxidation in biomembranes.

Free radical-induced deterioration of erythrocyte membranes and resulting hemolysis serve as a good model for studying the oxidative damage in biomembranes (16–26). It

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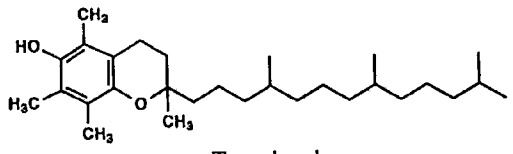
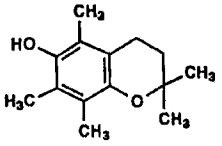
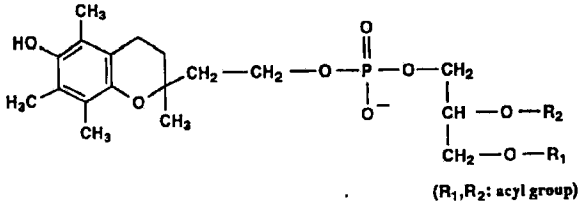
Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; PCh, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′8′-tetramethyl-6′-hydroxychroman; PL-OOH, phospholipid hydroperoxides; PMC, 2,2,5,7,8-pentamethyl-6-chromanol.



α-Tocopherol



PMC



PCh

SCHEME 1

has been well studied that free radicals attack erythrocyte membranes to induce peroxidation of lipids and proteins and eventually cause hemolysis (18–23). Therefore, the oxidative hemolysis of erythrocytes induced by free radicals is a well-defined model to estimate the antioxidative action of α-tocopherol and its analogs in biomembranes. In the present study, human erythrocytes were oxidized by a water-soluble radical generator, and the protective effect of PCh on the lipid peroxidation and hemolysis was investigated and was compared with those of α-tocopherol and PMC.

MATERIALS AND METHODS

Chemicals. 2,2′-Azobis(2-amidinopropane)-dihydrochloride (AAPH) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) were obtained from Wako Pure Chemical Co. (Osaka, Japan). *dl*-α-Tocopherol and PMC were obtained from Eisai Co. (Tokyo, Japan). Probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade. PCh was synthesized enzymatically from 2,5,7,8-tetramethyl-6-hydroxy-2-(hydroxyethyl)chroman and phosphatidylcholine and purified as described previously (11).

Preparation of human blood plasma and erythrocytes. Blood was taken from healthy volunteers and then was put into tubes containing EDTA, disodium salt (1 mg/mL). Plasma and erythrocytes were separated by centrifugation at 1000 × *g* for 20 min at 4°C. Erythrocytes were washed three times with 5 vol of the phosphate buffered saline (PBS). The buffy coat was carefully removed with each wash. At the last washing, the cells were centrifuged exactly at 1000 × *g* for 20 min to obtain packed cells with a constant volume.

Assay for oxidative hemolysis. Oxidative hemolysis of erythrocytes was performed as reported by Miki *et al.* (22) with a slight modification. Washed erythrocytes (0.5 mL) were suspended in 4 vol of PBS. Antioxidants, except for PCh, were added to the cell suspension as ethanol solution. The final ethanol concentration in the suspension was less than 0.5% (vol/vol), which had no effect on erythrocytes. Since PCh was only slightly soluble in ethanol, it was dispersed in PBS with a vortex mixer for 1 min followed by ultrasonic irradiation with an ultrasonicator (Model B1210J-DTH; Branson, Tokyo, Japan) for 30 s and then added to the erythrocyte suspension. After preincubation of this suspension at 37°C for 5 min, 2.5 mL of 150 mM AAPH dissolved in PBS was added to the suspension. Oxidation was carried out in the dark at 37°C under air with continuous shaking. When erythrocytes were oxidized after pretreatment with antioxidants, washed erythrocytes (1 mL) were suspended in 9 vol of PBS. Then antioxidant was added at the concentration of 50 μM in the suspension. After preincubation of this suspension at 37°C for 30 min, erythrocytes were washed three times with 10 mL of PBS. Washed erythrocytes were oxidized by the same aforementioned procedure. In both cases, oxidation was carried out in the dark at 37°C under air with continuous shaking. At specific intervals, the reaction mixture (0.1 mL) was withdrawn, diluted with 4 vol of PBS, and centrifuged (2000 × *g* for 5 min). The absorption of the supernatant at 540 nm was measured (absorption A). Similarly, the reaction mixture (0.1 mL) was treated with 4 vol of 0.1% Triton X-100 solution to yield complete hemolysis, and the absorption of the supernatant after centrifugation was measured at 540 nm (absorption B). Percentage hemolysis was calculated from the ratio of the readings, (absorption A/absorption B) × 100.

Oxidation of ghost membranes. Ghost membranes were prepared from erythrocytes by the method of Dodge *et al.* (27), after 25 mL of erythrocyte suspension (10%, vol/vol) was preincubated with each antioxidant (50 μM) at 37°C for 30 min, followed by washing three times with PBS (25 mL). The phospholipid concentration in the ghost membrane was calculated from the phosphorus content according to the method of Bartlett (28). Ghost membranes were suspended in PBS containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) at the concentration of 1 mM. The ghost membrane suspension (0.5 mL) was preincubated at 37°C for 5 min. Oxidation was initiated by adding 80 mM AAPH (0.5 mL) dissolved in PBS containing 0.5 mM DTPA. The reaction mixture was incubated at 37°C with continuous shaking in the dark. After 4 h of incubation, the reaction mixture (0.1 mL) was withdrawn and 0.1 mL of 10 mM BHT in ethanol was added. The lipids were extracted three times by the method of Bligh and Dyer (29). The lipid portion was evaporated with nitrogen gas, and the residue was dissolved in the mixture of methanol and water (95:5, vol/vol, 0.1 mL) for high-performance liquid chromatographic (HPLC) analysis of phospholipid hydroperoxides (PL-OOH) (30). Sample (10 μL) was injected into an octylsilyl column (TSK gel OCTYL-80Ts column, 6 × 150 mm; TOSOH, Tokyo Japan) and eluted with methanol/water (92.5:7.5, vol/vol). The hydroperoxides were

detected by ultraviolet absorption at 235 nm. The concentration of PL-OOH was calculated from the standard curve of phosphatidylcholine hydroperoxides. Standard phosphatidylcholine hydroperoxides were prepared as described previously (30).

Determination of antioxidants in erythrocytes and ghost membranes. After preincubation of 15 mL of erythrocyte suspension (10%, vol/vol) with each antioxidant (50 μM) for 30 min at 37°C, erythrocytes were washed three times with PBS (15 mL) and centrifuged at 1000 × *g* for 20 min. Then a hexane/ethanol mixture (4:1, vol/vol, 4 mL) containing 25 μM BHT and water (2 mL) was added to washed erythrocytes (0.5 mL). They were mixed with a vortex mixer for 30 s and were sonicated for 1 min followed by centrifugation at 1500 × *g* for 5 min. Hexane layers obtained from repeated extraction for three times were collected and evaporated with nitrogen gas. The residue was dissolved in the mixture of acetonitrile and chloroform (6:4, vol/vol, 0.1 mL) for the analysis of α-tocopherol, or in *n*-hexane (0.1 mL) for the analysis of PMC and PCh. α-Tocopherol was quantified by HPLC on an octylsilyl column (TSK gel OCTYL 80Ts, 4.6 × 100 mm; TOSOH) with acetonitrile/water (99:1, vol/vol) as an eluent at a flow rate of 1.2 mL/min. PMC was determined by HPLC on an aminopropylsilyl column [YMC Pack A-612(NH_2), 4.6 × 100 mm; Yamamura Chemical Laboratories, Kyoto, Japan] with hexane/isopropanol (99:1, vol/vol) as an eluent at a flow rate of 1.0 mL/min. For HPLC analysis of PCh, an aminopropylsilyl silica column (Shim-Pack FLC-NH_2, 4.6 × 50 mm; Shimadzu Co., Kyoto, Japan) was used with hexane/isopropanol/10% phosphoric acid (70:30:0.5, by vol) as an eluent at a flow rate of 2.0 mL/min. In each determination, the eluent was monitored fluorometrically at an excitation wavelength of 298 nm and an emission wavelength of 325 nm using a Shimadzu RF-10A (Shimadzu Co.). The respective concentration was calculated from the standard curve of the authentic compounds.

Measurement of protein. Protein was determined by the method of Bradford (31) using bovine serum albumin as a standard.

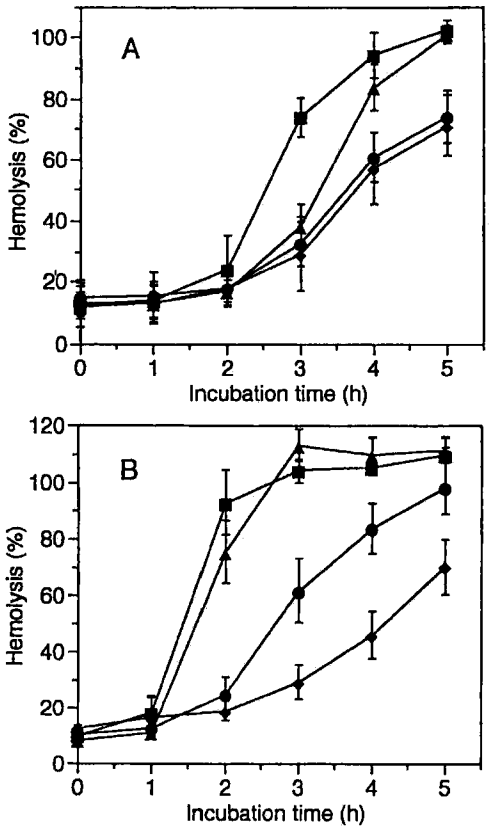


FIG. 1. Effect of coincubation (A) and preincubation (B) of erythrocyte suspension with α-tocopherol, 2,2,5,7,8-pentamethyl-6-chromanol (PMC), or 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman (PCh) on 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH)-induced oxidative hemolysis of erythrocytes. (A) Erythrocyte suspension (10%, vol/vol) in phosphate buffered saline (PBS) (pH 7.4) was incubated with AAPH (75 mM) and α-tocopherol or α-tocopherol analogs (50 μM). (B) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with α-tocopherol or α-tocopherol analog (50 μM) for 30 min followed by washing three times. After washing, the suspension was incubated with AAPH (75 mM). Each point represents the mean ± the standard deviation of three separate experiments. (■), No addition; (●), α-tocopherol; (▲), PMC; (◆), PCh.

RESULTS

Effects of antioxidants on free radical-induced hemolysis of human erythrocytes. Oxidative hemolysis was induced by a water-soluble azo compound, AAPH. Incubation of erythrocyte suspension with 75 mM AAPH at 37°C resulted in time-dependent hemolysis with an induction period (Fig. 1). In order to estimate the antihemolytic potency of α-tocopherol, PMC and PCh, erythrocyte suspensions were coincubated with each compound (50 μM) and AAPH (75 mM). α-Tocopherol and PMC were added to erythrocyte suspension as ethanol solution, while PCh was added as aqueous dispersion in PBS. These compounds retarded the occurrence of hemolysis and suppressed its percentage (Fig. 1A, coincubation experiment). α-Tocopherol and PCh exhibited nearly the same inhibitory effects and were more efficient than PMC. In a separate experiment, erythrocyte suspension was oxidized after pretreatment with each compound for 30 min, followed by washing three times to avoid the interference by unincorporated antioxidants (preincubation experiment). In this case, PMC had no protective effect (Fig. 1B). In contrast to PMC, α-tocopherol and PCh protected against hemolysis significantly even after pretreatment followed by washing. It should be noted that PCh was much more effective than α-tocopherol in suppressing the hemolysis of erythrocytes in the preincubation experiment, although little difference was observed between the two compounds in the coincubation experiment.

In addition to α-tocopherol analogs, we investigated the protective effects of BHT and probucol on oxidative hemolysis of erythrocytes in the preincubation experiment (Fig. 2). When preincubation was carried out in the absence of antioxidant, most of the erythrocytes were subjected to lysis after

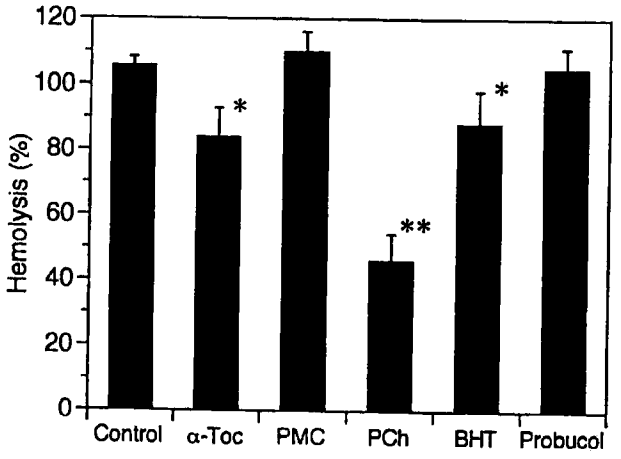


FIG. 2. Effect of preincubation of erythrocyte suspension with α-tocopherol, PMC, PCh, BHT, or probucol on AAPH-induced oxidative hemolysis of erythrocytes. Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with each antioxidant (50 μM) for 30 min followed by washing three times. After washing, the suspension was incubated with AAPH (75 mM) for 4 h. Bars represent the mean ± the standard deviation of three separate experiments. Asterisks indicate significant differences between control and antioxidant treatment: *, $P < 0.05$; **, $P < 0.01$. See Figure 1 for abbreviations.

incubation with AAPH (75 mM) for 4 h (control experiment). Probucol showed no significant protection similar to PMC, while α-tocopherol and BHT lowered the hemolysis to 83.8 ± 9.0% and 88.1 ± 9.8%, respectively. PCh exhibited a remarkable resistance to hemolysis, and the percentage of hemolysis was 46.0 ± 8.3%.

Incorporation of α-tocopherol, PMC, and PCh into human erythrocytes and their ghost membranes. In order to clarify the reason for the marked difference in antihemolytic potency among the three α-tocopherol analogs, the amounts of α-tocopherol and its analogs incorporated into erythrocytes and those accumulated within ghost membrane were measured after incubation for 30 min at 37°C (Table 1). Endogenous α-tocopherol was found at the level of 0.2 and 2.7 nmol/mg protein in erythrocytes and erythrocyte membrane fraction, respectively. The presence of endogenous α-tocopherol is likely to cause the induction period which was observed when erythrocytes without treatment of any antioxidants were exposed to AAPH (Fig. 1). When erythrocytes were incubated with α-tocopherol, PMC, or PCh at 50 μM, these compounds were incorporated into the cells at the concentration of 12.6 ± 0.7, 3.7 ± 0.2, and 16.3 ± 0.5 nmol/mg protein, respectively. In ghost membranes, PMC was not found, although α-tocopherol and PCh were found at 84.4 ± 6.2 and 101.0 ± 2.3 nmol/mg protein, respectively.

Effect of concentration of PCh in the pretreatment medium and pretreatment time on its antihemolytic activity. In order to study the antihemolytic action of PCh, the relationship between the concentration of PCh in the preincubation medium and its inhibitory effect against the hemolysis of erythrocyte was examined. The protective effect was increased with the

TABLE 1
Incorporation of α-Tocopherol Analogs into Erythrocytes[a]

Analog	Concentration of antioxidant (nmol/mg protein)			
	Erythrocytes		Ghost membrane fraction	
No addition		(0.19 ± 0.01)[b]		(2.68 ± 0.09)
α-Tocopherol	12.6 ± 0.7		84.4 ± 6.2	
PMC	3.7 ± 0.2	(0.21 ± 0.07)	n.d.[c]	(2.62 ± 0.10)
PCh	16.3 ± 0.5	(0.22 ± 0.03)	101.0 ± 2.3	(2.74 ± 0.13)

[a]Each value represents the mean ± the standard deviation of triplicate analyses.
[b]Values in parentheses are the concentration of endogenous α-tocopherol.
[c]Not detected. PMC, 2,2,5,7,8-pentamethyl-6-chromanol; PCh, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman.

elevation of the concentration up to 50 μM, reaching a plateau above 50 μM (Fig. 3A). The protective effect of PCh was enhanced with the increase of pretreatment time for up to 30 min, reaching a plateau for more than 30 min (Fig. 3B). Thus,

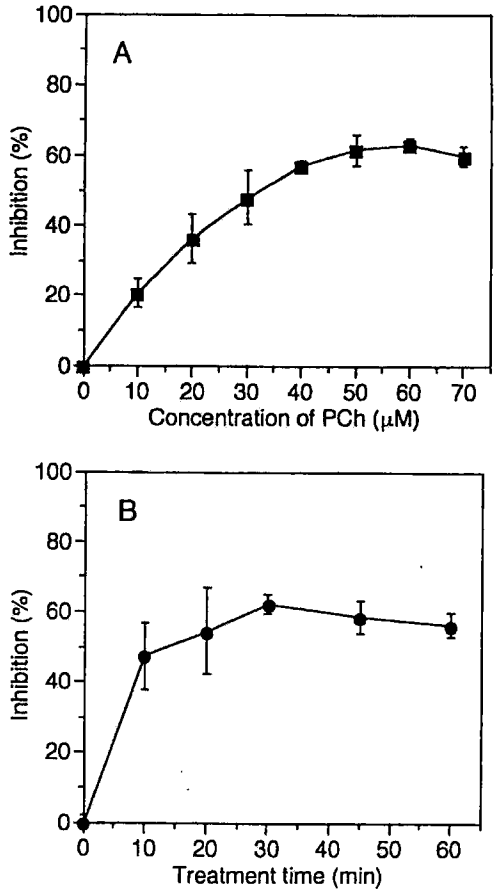


FIG. 3. Effect of the concentration (A) and the pretreatment time (B) of PCh on the protection of AAPH-induced oxidative hemolysis of erythrocytes. (A) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with PCh for 30 min followed by washing three times. (B) Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was incubated with PCh (50 μM) for specific time followed by washing three times. In both cases the suspension was incubated with AAPH (75 mM) for 4 h. Each point represents the mean ± the standard deviation of three separate experiments. See Figure 1 for abbreviations.

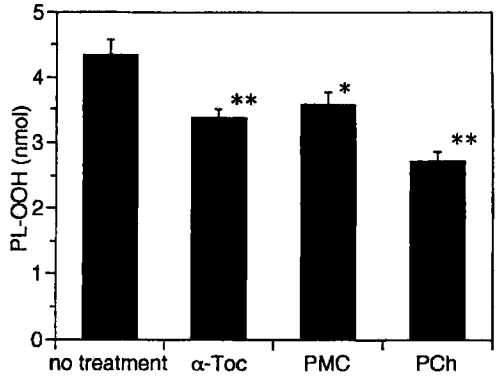


FIG. 4. AAPH-induced oxidation of α-tocopherol analog-pretreated ghost membranes. Erythrocyte suspension (10%, vol/vol) in PBS (pH 7.4) was preincubated with α-tocopherol, PMC, or PCh (50 μM) for 30 min, followed by washing three times, and then the ghost membranes were prepared by the method of Dodge *et al.* (27). The suspension of ghost membranes containing 0.5 μmol of phospholipids was incubated with AAPH (75 mM) for 4 h. Bars represent the mean ± the standard deviation of three separate experiments. Asterisks indicate significant differences between no treatment and α-tocopherol analog treatment: *, $P < 0.05$; **, $P < 0.01$. See Figure 1 for abbreviations.

an optimal concentration and an optimal time in the pretreatment of PCh appeared in the inhibition of oxidative hemolysis.

Oxidation of ghost membrane prepared from α-tocopherol-, PMC-, or PCh-pretreated erythrocytes. The ghost membranes prepared from erythrocytes after preincubation with each compound for 30 min were subjected to the oxidation initiated by AAPH. PL-OOH were accumulated as the primary products of ghost membrane peroxidation as shown in Figure 4. A lower amount of PL-OOH was accumulated in the pretreated ghost membranes. The membranes pretreated with PCh accumulated a lower amount of PL-OOH, as compared with those pretreated with α-tocopherol and PMC. This indicates that the resistance of erythrocyte membranes to free radical-induced lipid peroxidation was enhanced by the pretreatment of erythrocytes with α-tocopherol and α-tocopherol analogs, particularly PCh.

DISCUSSION

The above results show that PCh acts as a potent protector against oxidative cell injury in human erythrocytes and that its protective effect is superior to those of α-tocopherol and PMC. α-Tocopherol, PMC, and PCh are homologs of 2,5,7,8-tetramethyl-6-chromanol with phytyl side chain, methyl group, and phosphatidyl group, at the 2-position, respectively (Scheme 1). Although their structures are identical except for the substituents at the 2-position, their antioxidant effects seem to be much different from one another in a heterogeneous system because of their inherent physical properties.

We have reported that α-tocopherol, PMC, and PCh gave almost the same peroxyl radical-scavenging activity in homogeneous solution (11). On the other hand, α-tocopherol showed the longest induction period among the three compounds in phosphatidylcholine liposomes, probably owing to their different location in phospholipid bilayers (11,13,14), in which we have proposed that chromanol moiety in PCh is located at the interface between aqueous phase and lipid bilayers (13), analogous with the polar head group of phospholipids. It is reasonable that the difference in their antioxidant activities is, at least in part, derived from location and mobility of each analog in phospholipid bilayers. However, in intact cells the incorporation and retention of antioxidant within cellular membranes are also important factors in its activity. PCh is expected to possess high affinity for cellular membranes because of its nature as phospholipid that is a major component of cellular membrane bilayers. Therefore, this study is focused on the action of PCh in cellular membranes as compared with those of α-tocopherol and PMC.

The order of antihemolytic activity of the three compounds in the preincubation experiment appeared to be correlated with their amounts incorporated into erythrocytes. The local concentrations of α-tocopherol and PCh in erythrocytes were much higher than that of PMC (Table 1), indicating that α-tocopherol and PCh are effectively incorporated into erythrocytes. PMC could hardly be retained within the ghost membrane fraction in spite of the incorporation into the cells to some extent (32,33). These results indicate that phytyl chain and phosphatidyl moiety can act as an anchor to retain a chromanol moiety within membranes. α-Tocopherol and PCh incorporated into the membranes should play a role in preventing hemolysis by protecting the oxidation of membrane lipids. In fact, we found that the ghost membranes prepared from erythrocytes pretreated with α-tocopherol analogs elevated the resistance to free radical-induced lipid peroxidation (Fig. 4).

The existence of the α-tocopherol selective uptake system in red blood cells has been reported (6,7). Kaneko *et al.* (34) demonstrated that α-tocopherol was well incorporated into cultured cell because of the high affinity of its phytyl group with cell membranes. The fact that PCh was found in a higher amount than α-tocopherol in pretreated erythrocytes and their ghost membranes indicates that the affinity of phosphatidyl group with cell membranes is higher than that of phytyl group. Shuto *et al.* (15) reported that nucleoside analogs containing a phosphatidyl residue possess a high affinity for cell membranes, resulting in the penetration into cells. Furthermore, a phosphatidyl derivative of genipin, which is a water-soluble compound having some pharmacological activities, was found to exhibit the enhanced cytotoxicity in spite of the weak cytotoxicity of genipin itself (35). Therefore, it is concluded that a phosphatidyl group in PCh acts as an excellent carrier and anchor of chromanol group in erythrocyte membranes.

After the treatment of erythrocytes with α-tocopherol and PCh for 30 min, the concentrations of α-tocopherol and PCh reached similar levels, which were 66 and 74 times in the cells, and 31 and 37 times in the ghost membranes greater than endogenous α-tocopherol concentration, respectively (Table 1). However the inhibition of AAPH-induced hemoly-

sis by the treatment with PCh was much greater than that by the treatment with α-tocopherol (Figs. 1 and 2). Thus, the marked difference in antihemolyic potency between α-tocopherol and PCh cannot be fully explained by their cellular concentrations. Free radicals generated from AAPH are known to attack erythrocyte membranes from the outside to induce lipid peroxidation in membrane phospholipids (18–23). The chromanol moiety of PCh seems to be located at the interface of the membrane and scavenges aqueous radicals rather than lipophilic radicals efficiently, while α-tocopherol mainly scavenges chain-carrying lipid peroxyl radicals in the hydrophobic region of membranes (13,14). The ability of PCh in scavenging aqueous radicals seems to be of great advantage to suppress their attacks on erythrocyte membranes and resulting in hemolysis.

Enormous amounts, as compared to endogenous α-tocopherol, of α-tocopherol and PCh incorporated into ghost (Table 1) did not result in efficient inhibition of AAPH-induced formation of PL-OOH (Fig. 4) but prevented efficient hemolysis (Fig. 1 and 2). However, it might be possible that the exogenous antioxidant prevents not only lipid peroxidation but also protein oxidation that was also observed in AAPH-induced oxidation of ghost membranes (18,22), resulting in effective prevention of hemolysis (Figs. 1 and 2).

Although PMC was incorporated into erythrocytes to some extent, it was not retained in ghost membrane fraction (Table 1). PMC can not be anchored because of the lack of long chain and may be transferred freely without orientation in the membrane (9,35,36); hence it may penetrate into cytosol easily. No inhibition on the oxidative hemolysis by PMC appears to be due to inability to be retained within the membranes. Niki *et al.* (32) reported that the mobility of PMC was quite high within and between liposomal membranes. Furthermore, they have shown that the antioxidant activity of chromanols was elevated with decreasing length of side chain in the oxidation of plasma low density lipoprotein (36). Thus, PMC, having no long side chain, can transfer easily from erythrocytes to outside of cells, resulting in less effective oxidative hemolysis of human erythrocytes.

The efficiency of antioxidants in the heterogeneous phase is determined by not only their radical-scavenging activity but also their physical properties, such as local concentration and mobility in the microenvironment (36). The present results obtained from antihemolytic activity suggest that the ability both to be incorporated into the cells and to be retained within the membranes should be involved in the factors responsible for the action of antioxidant in biomembranes. It is clear that phosphatidyl moiety makes an antioxidant molecule easier to be inserted into cellular membranes. Phosphatidyl derivatives may be promising compounds in the prevention or cure of cellular oxidative damage-related diseases.

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